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(54) Non-instrumented cholesterol assay.

(57) An assay for determining the cholesterol level in a sample involving a bibulous strip comprising a transfer region for transporting a transport medium from a transport medium source, a sample receiving region in fluid communication with said transfer region, and a measurement region in fluid communication with said sample receiving region, and a detectable signal reagent system comprising unbound conversion reagent and bound reagent wherein said conversion reagent reacts with cholesterol to form an intermediate product and wherein said bound reagent reacts with said intermediate product to produce a detectable border, in which the conversion reagent is placed in the transfer region of the strip or in a region of the strip between the sample receiving and measurement regions and the signal reagent is non-diffusively bound to the strip in the measurement region, and which upon contact with the sample and the transport medium results in the production of a detectable border in the measurement region which is related to the level of cholesterol in the sample.

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NON-INSTRUMENTED CHOLESTEROL ASSAY

INTRODUCTION

Technical Field

The field of this invention is non-instrumented diagnostic devices and assays for determining cholesterol levels in samples.

Background

Cholesterol is a hydrophobic molecule found in all animals as an essential component of c ll membranes and hormones and is known to be involved in other vital functions as well. Since cholesterol is present in all animal tissue, cholesterol is consumed every time food of animal origin is eaten. Although cholesterol is essential for body processes, it is not necessary for this compound to be ingested, since the liver can produce all that is needed.

Cholesterol is found and stored in blood as a fatty acid ester which is complexed with serum proteins. These cholesterol ester-protein complexes found in blood are termed lipoproteins. In this way "nature" has found a method to allow water insoluble cholesterol to become soluble in whole blood. There are two types of lipoproteins which have been identified in blood, and these two types are low density lipoproteins (LDL) and high density lipoproteins (HDL). LDL cholesterol is known to contain a high proportion of cholesterol and has been indicted as being the agent responsible for the deposition of cholesterol in artery walls. HDL cholesterol, on the other hand, is believed to transport cholesterol to the liver for removal from the blood. Thus, LDL cholesterol has been characterized as "bad" cholesterol, while HDL cholesterol has been characterized as "good" cholesterol.

A relationship has been established between total blood cholesterol (which is primarily the LDL fraction) and coronary artery disease. Guidelines have been established for adults over 20 years of age to identify risk groups associated with blood cholesterol level. These levels are as follows: <200 mg/dl is desirable blood cholesterol; 200 to 239 mg/dl is borderline high blood cholesterol; >240 mg/dl is considered high blood cholesterol.

Cholesterol levels can be controlled by both diet and cholesterol-lowering drugs. The key to such efforts to control cholesterol levels is to identify those individuals at risk. There has been an effort in the past several years to identify individuals with elevated cholesterol levels and initiate treatment. This effort is expected to lower mortality from coronary heart disease. A procedure whereby cholesterol levels could be easily and conveniently determined at home, therefore, would be particularly useful in the effort to lower mortality from coronary heart disease.

The following methodology includes assay strip design and performance characteristics for a non-instrumented whole blood cholesterol assay which is well-suited for home use. This type of home testing will improve the method for identification of those at risk and further reduce death from coronary h art disease.

Relevant Literature

Demacker et al., Clin. Chem. (1983) 29:1916-1922 reports the evaluation of cholesterol assay kits. Studies associated with enzyme assays include Gochman and Schmitz, Clin. Chem. (1971) 17:12; Paul, The Enzymes (1963) 8:227-274; Current Status of Blood Cholesterol Measurement in Clinical Laboratories in the United States: A Report from the Laboratory Standardization Panel of the National Cholesterol Education Program (1988) 34(1):193-201, and U.S. Patent Nos. 4,391,904, 4,366,241, 4,168,146, 4,435,504, 4,533,629, 4,504,659, and the references cited therein. See also, Zuk et al., Clin. Chem. (1985) 31:1144-1150.

German Patent No. 22 951 describes a filter assembly containing chemical reagents for removing cells from blood and measuring CPK.

SUMMARY OF THE INVENTION

The present invention involves methods and apparatus for use in determining cholesterol levels in samples by using a continuous flow path assay in which the cholesterol is r acted with a conversion reagent to provide an intermediate product which in turn reacts with a bound reagent on the path to

produce a detectable signal having a detectable border whose distance from a predetermined site is related to the amount of cholesterol in the sample. Improved accuracy and sensitivity are obtained in the assay by placing the conversion reagent, and/or additional reagents needed to produc the detectable signal from th intermediate product and bound reagent, on the path upstream or downstream of the path region initially contacted with the sample, such that the reagents are transported by a transport medium through the bound reagent region.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagrammatic plan view of a measuring strip according to this invention;

Figure 2 is a diagrammatic plan view of the base plate and slide of an alternate embodiment according to this invention;

Figures 3a and 3b are diagrammatic plan views of an intermediate plate which covers the base plate, and of the plate inverted to show the underside, respectively;

Figure 4 is a planar view of an assembled device; and

Figures 5-8 are diagrammatic plan views of various embodiments of a laminated measuring strip according to the invention.

DESCRIPTION OF SPECIFIC EMBODIMENTS

Methods and apparatus are provided for the measurement of cholesterol in a sample employing a continuous flow path, which has a transfer region, sample receiving region, an optional detectable signal reagent capturing region, and a measurement region. The presence of cholesterol and conversion reagent of the detectable signal reagent system results in production of an intermediate product, which directly or indirectly reacts with another member of the detectable signal reagent system which is bound to the surface of the measurement region. The result is the production of a detectable signal, generally a colored zone, in the measurement region. The distance of the border of the detectable signal from a predetermined site can be related to the amount of cholesterol in a sample.

Various techniques may be employed for organizing the flow path and providing for flow of a reagent solution through the sample region, the reagent capturing region, and the measurement region. In addition, various chemistries may be employed, using different types of reagent systems to produce the desired signal. The assay may be semiquantitative or quantitative. For quantitative assays, the distance of the border of the detectable signal, i.e., colored border, from a predetermined site indicates the amount of cholesterol in the sample.

The general design of such assays is set forth in copending U.S. Patent Applications Serial No. 433,538 filed November 8, 1989, which application is a continuation-in-part of Serial No. 357,045, filed May 29, 1989, which application is a continuation-in-part of Serial No. 324,407, filed March 16, 1989, which is a continuation-in-part of Serial No. 195,881, filed May 19, 1988, and Serial No. 064,883, filed June 22, 1987, which disclosures are incorporated herein by reference in their entireties.

The flow path comprises several regions, where certain regions may be overlapping, in whole or in part: (a) transfer region, (b) sample receiving region, (c) optional capture region, and (d) measurement region. The device embodying the flow path may be of any suitable configuration, preferably a strip which has the various regions aligned in the direction of transport medium flow.

The transfer region receives the transport medium and initiates the flow of the transport medium into the flow path. For the most part, particularly with extended strips, it will be a bibulous short element which serves by capillary action to wick or transport the transport medium to the next region, normally the sample receiving region. For the most part, the transfer region will be a bibulous strip which absorbs a hydrophilic liquid and allows for transport of any reagents contained in the transport medium with or without chromatographing the components of the transport medium. Where the device is a circular disc, the transfer region will be at the center of the disc and allow for transport of the medium radially away from the center through the other regions.

The sample receiving region serves to receive theTMsample and to act as a bridge for transferring the transport solution to the next region. In one embodiment, prior to the time that the transfer region serves to transport the medium to the sample receiving element, adjacent regions to the sample receiving region will not be in fluid receiving relationship with the sample receiving region. In that embodiment, after receiving the sample, the sample receiving element is then permitted to be a bridging element which allows for the flow of the transport solution through the sample receiving region and into the next region.

The sample receiving region may take a number of forms but may be simply a site on the bibulous strip where the sample is placed. Alternatively, it may be a pad in fluid transferring relationship with an underlying strip with or without enzyme(s) to enzymetically convert cholesterol to produce hydrogen

with another member of the detectable signal reagent system bound to the continuous flow path or strip to produce a detectable, e.g., colored, signal on the path or strip. The detectable signal reagent system may also comprise additional reagents, such as other intermediate components or catalysts needed to produce a detectable signal. The conversion reagent preferably includes reagents which react with cholesterol esters and cholesterol to form hydrogen peroxide. Such reagents are most preferably cholesterol esterase (EC:3.1.1.13) and cholesterol oxidase (EC:1.1.3.6). The serum cholesterol ester is hydrolyzed by the cholesterol esterase, and subsequent oxidation of the cholesterol is accomplished by the cholesterol oxidase to produce a stoichiometric amount of hydrogen peroxide. The hydrogen peroxide can in turn react with a peroxidase substrate on the continuous flow path in the presence of horseradish peroxidase to form a colored region on the path, which region relates to the level of cholesterol in the sample. The cholesterol esterase can be immobilized at 5 to 50 units/ml most preferably at 18 units/ml. The cholesterol oxidas can be immobilized at 10 to 100 units/ml, most preferably at 50 units/ml. The horseradish peroxidase can be immobilized along the flow path of the assay strip at immobilization concentrations 0.05 to 2.5 mg/ml, usually at 0.5 mg/ml. Peroxidase may also be included in the transport medium at 0.0005 to 0.050 mg/ml.

By appropriate choice of members of the detectable signal reagent system, visually observable color fronts, fluorescent signals, or the like may be obtained for a quantitative assay.

The conversion reagent of the detectable signal reagent system is placed in the continuous flow path in the transport region, the sample receiving pad or the region between the sample receiving and measurement regions, preferably in the region between the sample receiving and measurement regions. Upon movement of the transport medium through the path, the conversion reagent is transported into contact with the sample or vice versa depending upon whether the conversion reagent is placed upstream or downstream of the sample receiving region. Any additional reagents of the detectable signal reagent system, other than the bound reagent in the measurement region, may be a part of the transport medium or placed in the continuous flow path in the transport region or the region between the sample receiving and measurement regions, preferably in the region between the sample receiving and measurement regions. The transport medium transports these additional reagents through the path.

Other reagents may also be present in the assay. For example, detergents find particular use in the present assay to minimize or eliminate the binding of cholesterol to other proteins in the sample. Thug, detergents such as non-ionic, anionic, or cationic detergents may be employed. Of particular interest are polyoxyalkylenes, ethoxylated alkylphenols, octylphenoxypolyethoxy-ethanol, octylphenol-ethylene oxide condensates and polyoxyethylene lauryl ethers, or anionic detergents, such ag bile acids, e.g., sodium cholate and sodium taurocholate. In addition, various sticking agents or adhesives may be employed, such as gum arabic. Also of interest will be proteins which are substantially non-interfering, which may include gelatin, casein, serum albumin, or gamma-globulins. In addition, the reagent containing regions may include preservatives, such as sucrose, polyvinyl alcohol, polyvinyl pyrrolidone, dextran, or sodium azide. Finally, a buffered solution will normally be employed for impregnating the reagent containing regions. Any convenient buffer may be employed, preferably a substantially dilute buffer, which may include phosphate, TRIS, MOPSO, borate, carbonate, or the like. Usually, the buffered solution will be at a pH in the range of about 4 to 9. The buffer concentration will generally be from about 10 to 500 mM.

The conversion medium applied to the chip typically will contain about 0.1 to 5 weight percent detergent. In the case of detergent mixtures, the weight of non-ionic detergents may be about 10 to 90 weight percent, usually about 25 to 75 weight percent, of the total detergent mixture. The binding agents or adhesives will generally be in the range of about 0.2 to 10 weight percent, more usually about 1 to 5 weight percent, of the medium. A preservative or hydrogen bonding agent may be present in about 1 to 20 weight percent, more usually about 2 to 10 weight percent. The remaining additives will generally be present in total amount of less than about 10 weight percent, more usually of less than about 5 weight percent. The remaining composition may be water, non-reactive ingredients, excipients, extenders, and the like.

The assay is carried out by impregnating a sample receiving region with the sample. In a preferred embodiment, the sample receiving region is a pad, which serves as a bridge between the other flow path elements positioned in tandem juxtaposition along their long axes. Thus the elements define one long flow path, usually comprised of differently sized bibulous strips or areas, conveniently with a separation between strips, where the sample receiving element may act as a bridge to allow for fluid flow between strips.

Blood will be the sample typically assayed for cholesterol. The sample receiving region, therefore, is preferably positioned under a red blood cell removing filtering device. The blood sample will normally be one or a series of small drops, generally having a total volume under about 100 '"m"'l, more usually about 10-50 '"m"'l. The layers through which the sample flows will usually include a mesh layer, a first membrane, and a second membrane cooperating with the first membrane to ensure the substantially complete removal of any interfering cells from the blood sample. The first cellular separation member is

used to reduce the concentration of red and white blood cells received by the second filtration member. By lowering the red blood cell content about 10 to 90%, usually about 30 to 90%, of the original red blood cell content with the first membrane member, the second membrane member is able to efficiently and accurately remove at least substantially all of the red blood cells from the blood sample. Since the first membrane acts as a coarse separation means, the first membrane may take any of a wide variety of forms.

Various packings or sieving depths of filters may be employed, such as glass fibers, cellulose filters treated with red blood cell capture reagents, glass fiber filters, or synthetic fiber filters. Glass fiber filters are available from such manufacturers as Whatman, Schleicher and Schuell, MSI, and Pall. The glass fiber filters are further characterized by a glass fiber diameter in the range of about 0.5-9 '"m"', and a density of about 50 to 150 g/m^2. The glass fiber filters may be illustrated by S&S Glass 30, Whatman GFD, and S&S 3362.

Other coarse separation membranes may include cellulosic membranes, e.g., filter paper, to which red blood cell binding proteins or agglutination agents are immobilized. Such proteins may include lectins, antibodies specific for RBC surface membrane proteins, thrombin, ion exchange agents, etc. The preparation of such filters by conjugating proteins or other agents to cellulose is well known. Cellulose may be activated in a wide variety of ways employing carbodiimide, carbonyl diimidazole, cyanogen bromide, chloroacetic acid, where the acid may then be activated with carbodiimide, or the like. The literature is replete with examples of binding of proteins to cellulosic membranes for a variety of reasons, which techniques may be employed here. Alternatively, multiple layers of coarse separation membranes may be employed.

When two membranes are used, the second membrane will preferably be immediately beneath the first membrane and will be in fluid receiving relationship with the first membrane, either in contact with the first membrane or in close proximity thereto. Generally, the spacing between the first and second membranes will not exceed a distance which inhibits fluid flow, so that fluid readily flows from the first to the second membrane. The non-asymmetric membranes which are employed will be those in the medium porosity range, having an average porosity in the range of about 0.65 '"m"' to 7 '"m"', preferably about 1 to 5 '"m"', where the pores may or may not be of substantially uniform diameter through the membrane. By contrast, where an asymmetric membrane (i.e., one wherein the diameter of the pores vary from one surface to the other) is employed, desirably the membrane will have a minimum porosity not less than about 0.2 '"m"', preferably not less than about 0.45 '"m"', and the maximum porosity will generally not exceed about 40 '"m"', more usually not exceeding about 20 '"m"'. Illustrative microporous membranes which may find use include Filterite polysulfone asymmetric, 20 '"m"' - .45 '"m"'; Sartorious cellulose acetate, 1.2 '"m"'; and Nucleopore 1.0'"m"'.

The choice of the second membrane is important, since the amount of red blood cell lysis is dependent on a number of factors. Depending on the size of the pores, the amount of lysis will greatly vary. Since lysis results in release of colored cell components, which interfere with detection of the border in the measuring strip and may act to decompose detectable signal reagent system components, particularly hydrogen peroxide, merely removing cells is insufficient. A further consideration is the pressure differential across the membranes. Again, the appropriate choice of membranes will affect the pressure drop and forces acting on the cells, which in turn can affect the stability of the cells.

Thus, the two membranes serve to act together to efficiently and accurately remove red blood cells from the blood sample with little, if any, hemolysis, so as to provide a plasma or serum sample which may be accurately analyzed without interference from hemolytic products, such as heme.

The sample receiving region will be immediately beneath the red blood cell removing membranes and in fluid receiving relationship with the membranes. The sample receiving region will normally be a bibulous member able to absorb the fluid. Various bibulous materials may be used, such as cellulosic materials, e.g., paper, or the like. The sample receiving region will usually be of a size in the range of 5 to 100 mm^2 surface area, usually not more than 50 mm^2 surface area, and a thickness in the range of about .1 to 2 mm, having a volume capacity of from about 1 to 75 '"m"'l. When the sample receiving region is a pad, the pad may be round, square, rectangular, quadrilateral, or polygonal, depending on the manner in which it is to be used to act as a bridge for the other members of the flow path. For further characterization, see copending U.S. patent application Serial No. 195,881, filed May 19, 1988.

The applied sample will be absorbed in the sample receiving region and may extend outside the region, both upstream and downstream, depending upon the size of the region, the nature of the assay, and the nature of the upstream and downstream regions. In one embodiment, the sample is prevented from interacting with the adjacent bibulous members when sample is transferred to the sample receiving region.

Various techniques may be employed to prevent transfer of the sample from the sample receiving region (e.g., a pad) to the other regions prior to flow of the transport medium. Of particular interest is the

use of a slide which can be moved from a first position, where the sample receiving region receiv s the sample, to a second position where the sample receiving region serves as a bridge between the two other regions of the flow path. The slide therefore prevents sample from spreading to the other regions of the flow path, before it is time to carry out the assay. The path of the sample receiving region, in moving from the site at which the sample is received to the site where it is in the flow path, may provide for means for removing excess sample from the sample receiving region. Such means provides for a quantitative measur of the amount of sample received by the sample receiving region. Thus, by having a region in the path of the slide which is narrowed, so as to remove unabsorbed sample medium, without significantly squeezing the sample receiving region, the amount of sample absorbed by the sample receiving region can be relatively accurately reproduced. The narrowing may be as a result of a convexity, such as a rod in relief, a roller, or any convenient scraping means. The narrowing of the path should provide a space about equal to or slightly less than the wet thickness of the sample receiving region. The slide, therefore, not only serves to move the sample receiving region, but also to meter the amount of fluid absorbed by the sample receiving region.

Besides the slide mechanism, other flow inhibition means may be employed, usually comprising an inert non-porous film, which blocks transfer from the sample receiving element to the bibulous members of the flow path. The amount of sample accepted by the sample receiving element and involved in the assay medium may be controlled by providing for transfer of fluid beyond the amount saturating the sample receiving element through a non-wetting screen into an absorbent layer. After addition of the sample to the sample receiving element, and an incubation of up to about 30 minutes, the porous non-wetting material and absorbent layer are removed, leaving the sample receiving element as the sole repository of sample for the assay. Where a wiping film is employed it will be removed upon saturation of the sample receiving element. (See U.S. patent application Serial No. 324,407, filed March 16, 1989.)

The entire flow path may have a length of about 25 to 200 mm, more usually from about 50 to 150 mm, preferably about 100 mm. About 25 to 90% of the length of the flow path will be the measurement region comprising the quantitation zone, optionally a mixing region and/or a threshold value region. The mixing and/or threshold value regions will generally be about 5 to 35% of the flow path. The strips which provide for flow of fluid to and from the sample receiving element may be of the same or different length and each will generally be from about 5 to 25 mm, more usually about 10 to 20%, of the length of the flow path. The upstream strips may be part of the measurement region strip, or independent entities. Alternatively, this strip may be used to control the threshold value. The sample receiving region will generally be from about 1 to 10%, more usually from about 2 to 8% of the length of the flow path; the longer the flow path, the larger the sample receiving region may normally be. The width of the strips may be varied, usually being at least about 2 mm and not more than about 10 mm, preferably about 3 to 7 mm.

Any convenient material may be used for the various bibulous parts of the assay strips forming the flow path. Usually, the thickness of the bibulous components will be in the range of about 0.05 to 2.0 mm, more usually 0.15 to 0.75 mm. A wide variety of bibulous supports may be employed, particularly cellulosic supports, such as chromatography paper, silica on a support, alumina on a support, and polymeric membranes such as nitrocellulose and nylon. The characteristics of the bibulous material employed for the measurement region include the need in many instances to covalently or irreversibly bind an indicator molecule to the support, to develop a clear and sharp color, and to ensure that the fluid is capable of flowing at a convenient rate through the bibulous members.

Of particular interest is an assay device which is self-contained and only requires the sample for carrying out the assay. The device may serve as a one-step diagnostic test device using a disposable cassette format. The device may be fabricated of three individual injection molded parts into which various components of the assay system are associated. These include the filtration medium designed to separate plasma from whole blood, means for metering a precise sample volume, a glide to transfer the sample receiving element to the transfer and measurement elements and to release a transport and reagent solution. The transport solution initiates capillary migration through the flow path resulting in the development of a detectable boundary related to the amount of analyte in the sample.

Where a reduced amount of the product resulting from reaction of the cholesterol with an enzyme is present in the measuring region due to capture of such product in the capture region, it will frequently be desirable to have a lower concentration of members of the detectable signal reagent system than would be used in the absence of the capturing region. For example, where hydrogen peroxide is the product in a cholesterol assay, the optimum amount of non-diffusively bound measurement reagent such as a substitute for horseradish peroxidase is in the range of about 0.25 to 0.50 mg/ml in the dip immobilization solution, while in the present embodiment using a capturing region the dip concentration of peroxidase substrate may be lowered by 10 to 75% to a range of 0.05 mg/ml to 0.45 mg/ml.

Th subject invention is now considered in light of the drawings which depict several pref rred embodiments of the present invention. In a preferred embodiment in Figure 1, a strip 10 is provided which has a scored area 12 which includes pad 14 as the sample receiving element for receiving the sample. Pad 14 may be removed from the scored area 12 and dipped in the sample to provide for a semiquantitative measurement of the amount of sample absorbed by the sample receiving element 14. After dipping the sample receiving element into the sample, it is then returned to the scored area 12 and firmly fitted into the scored area so as to be part of device 10. The region below, i.e., upstream, from the sample receiving region is the transport region which is used to wick up the transport medium.

Downstream from the sample receiving element 14 is conversion reagent region 16. In this region, for™ example, one would have cholesterol esterase and cholesterol oxidase for reacting with the cholesterol to produce hydrogen peroxide.

Downstream from the conversion reagent region is the capture region, a narrow zone which serves to capture a predetermined amount of a component of the detectable signal reagent system which is present in limited amount as a result of being present in an amount related to the amount of cholesterol in the sample. In this situation, again using hydrogen peroxide production as illustrative, one could use a leuco dye which reacts with hydrogen peroxide in the presence of peroxidase. Thus, in region 18, one would produce a deep color if cholesterol is present in excess of a threshold value determined by the amount of dye in region 18. This dye could be spotted and dried at the base of region 20 thus eliminating the need for a discrete region and producing a similar step-gradient of reactive dye along the flow path of the assay strip. Region 20 would have a much lower density of dye throughout the region so as to allow for production of a border, where one can detect the end of the reaction between the hydrogen peroxide and th dy . Desirably a rocket as depicted by broken line 22 is obtained, where one can clearly delineate the top of the rocket from the region immediately upstream from line 22. Desirably, the side edges 24 are perforated, since this appears to provide for a sharper delineation of the border. See U.S. Patent No. 4,757,004.

A more sophisticated device is shown in Figures 2 to 4. The invention may be fabricated from three injection molded parts or by any other convenient process. The parts comprise a base plate 40, a slide 42 and a cover plate 44, as shown in Fig. 2 and 3. The base plate 40 consists of a cutout to accept the glide 42, a slot 46 with locating pins 48 into which the quantitation strip 50 and bibulous strip 52 are precisely positioned, maintaining about a 2 mm gap 54 between them, and a well 56 designed to capture the released transport solution, e.g., wicking buffer.

The slide 42 consists of a vented receptor site 58 into which the sample receiving pad 59 is inserted, an arm 60 with shearing action designed to facilitate the release of the transport solution from a pouch which is housed in well 62 of cover plate 44, and a snap 61 to lock the slide in place, once pulled. The sample receiving pad may have antibodies which react with the cholesterol to prevent the cholesterol from reacting with detectable signal reagent system components. Thus, for example, a predetermined amount of cholesterol can be inhibited from reacting with enzyme to produce hydrogen peroxide.

The cover plate 44 consists of a well 62, which houses a sealed foil pouch (not shown) containing the transport solution or the well 62 may be filled with transport solution and covered with a peelable foil seal. The cover plate has an orifice 64 for the introduction of the sample. Underneath orifice 64 are filters 66, for separating cells from blood samples. The filtration system may comprise dual glass fiber disks and a final filtration membrane in order to deliver cell free plasma to the sample receiving element. The cover plate also comprises the squeegee metering bar 68, which serves to control the volume of sample absorbed by the sample receiving element, as well as a viewing slot 70. At the top of the viewing slot 70 is an indicator hole 72, which changes color when the test is complete to inform the user that a reading may be taken.

The final assembly is depicted in Fig. 4, where the assembled device is obtained by introducing the slide 42 into base plate 40, positioning transfer strip 52 and measurement strip 50 at their appropriate sites, introducing the transport solution pouch into well 62 or as indicated above, filling well 62 with transport solution and sealing with a Poly-foil seal, assembling the cover plate and base plate and then sealing, conveniently by sonic welding, the base plate and the cover plate. This procedure locates the sample receiving site of the slide directly beneath the filtration media of the cover plate, as well as locating the shearing points of the slide beneath the foiled sealed pouch located in the cover plate.

In order to carry out a cholesterol measurement, the user lances a finger and applies a hanging drop of blood to the application site, which is a white central well with a red or black border. When the white center is no longer visible, a sufficient amount of blood has been applied. A fill to line may also be included which is located about 0.5 to 1mm above the filters around the bottom of the sample well. When the sample covers the line, a sufficient amount of blood has been applied. The user then waits about 30 seconds to 2 minutes or more to allow adequate filtration and recovery of plasma onto the sample receiving pad.

The slide is then pulled until it snaps into place. At this point the sample receiving pad containing th

plasma sample has been metered by the squeegee metering bar and is brought into contact and fluid transferring relationship pith the transfer region and the measurement region or the reagent region bridging under the two mm gap. The shearing points of the slide have also pierced the foil seal of the pouch in the well of the cover plate, releasing th transport solution into the receiving well in the base plate. The transport solution is 0.10 to 2 ml of an aqueous buffer which may contain horseradish peroxidase in sufficient amount to insure rapid reaction of the hydrogen peroxide and dye or the horseradish peroxidase may be present diffusibly bound on the strip either upstream or downstream of the sample receiving region. Upon contact of the strip with the transport solution, the strip assembly begins wicking up the transport solution which washes the cholesterol esterase and cholesterol oxidase enzymes into the sample, or vice versa depending upon the reLative placement of enzymes and sample, and further washes the hydrogen peroxide reaction product of the enzymes and cholesterol, as well as the horseradish peroxidase, into the measurement region of the strip.

The measurement region is impregnated with a peroxidase substrate, particularly a modified N,N-dimethylaniline. (See U.S. patent application Serial No. 195,881, filed May 19, 1988.) The reaction of the reagents results in a colored region with a defined boundary, thereby giving the user a precise reading of the cholesterol level above a threshold level. This reading is made when the color indicator site above the viewing slot shows the test is complete. Normally, it will take fewer than about 15 minutes for the assay to be complete, reading the peak of a colored area in the viewing slot.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

EXAMPLE 1

This example illustrates the separation between cholesterol levels that can be obtained through placement of the cholesterol conversion reagent on the strip in the transfer, in the region between the sample receiving and measurement regions on the sample receiving or in the transport solution.

In carrying out the assay for cholesterol, a device was prepared having an overall length of about 95mm; 12mm of which was the transfer region, about 7 mm was for the sample receiving pad, and the remainder was the measurement region. The strip was made of Whatman 31ET paper, where a modified N,N-dimethylaniline was covalently linked to the paper with MBTH being immobilized passively in the measurement region. This strip configuration was used when the cholesterol conversion reagents are on the sample receiving pad, the transfer region or in the transport solution. When the conversion reagents are positioned between the sample receiving and measurement regions, an additional strip area is included directly upstream from the sample receiving area. This strip area is 7mm long as seen in Fig. 8 and is called the conversion pad.

The conversion solution comprised 5.33 g sodium phosphate dibasic, 1.25 g sucrose, 1 ml Nonidet P-40, 1.0 g cholic acid, 0.83 g Mega-8, 0.71 g sodium potassium tartrate, 0.65 g sodium nitroprusside, 0.577 g sodium stannate, and 0.01 g sodium azide, after the addition of which the solution was adjusted to pH 7.0, and 1800 units of cholesterol esterase and 5000 units of cholesterol oxidase added thereto, and the volume brought to 100 ml. The strip was wetted with this solution in the conversion region and dried. Each test contains about 0.18 units cholesterol esterase and 0.60 units of cholesterol oxidase.

The wicking buffer or transport solution was comprised of 0.05 M sodium phosphate, 2 mg/ml BGG, 0.005 mg/ml HRP, 0.01% gentamycin, and 0.01% Nonidet P-40.

The resulting device was tested using three calibrators at different levels provided by the College of American Pathologists (CAP), with cholesterol levels at 137, 234 and 333 mg/dl which use lyophilized human serum. Calibrators were reconstituted fresh each day.

Four modifications of the above device were tested for separation (in mm) between the tri-level CAP calibrators. The device modifications involved the location of the conversion reagents: (A) sample receiving region, (B) transport solution, (C) transfer region, and (D) region between the sample receiving and measurement regions.

In carrying out the assay using the above four device configurations, a 10 '"m"'l serum sample was placed on the sample receiving pad, and the transport solution allowed to wick up the strip. The cholesterol reacts with the conversion reagents, specifically the cholesterol esterase and cholesterol oxidase to form hydrogen peroxide, which in turn reacts with the MBTH in the presence of the horseradish peroxidase solution to produce a blue color. After the wicking was complete, the color front height was measured, which color front height is directly correlatable with the serum cholesterol level.

With the above modified designs, separation improved to provide from 6.6 to 8.7 mm between each 100

mg/dl change. This compared with a 4.5 to 7.2 mm separation between every 100 mg/dl change using the configuration in which the conversion reagents were placed in the sample receiving region. Overall, there was observed a 27.5% increase in separation over the full assay range of about 200 mg/dl with the configurations in which the conversion reagent was placed in the transfer region or in the region between the sample receiving and measurement regions, as compared to the configuration in which the conversion reagent was placed in the sample receiving region. The following table summarizes the results.

TABLE 1

Location of Conversion Reagents	Cholesterol (mg/dl)[1]			Signal Separation (mm)		
	137	234	333	137-234	234-333	137-333
A. Sample Receiving Region	27.3	34.0	41.2	6.7	7.2	13.9
B. Transport Solution	34.5	42.0	46.5	7.5	7.5	12.0
C. Transfer Region	26.0	34.7	41.3	8.7	6.6	15.3
D. Region Between Sample Receiving and Measurement Regions	26.0	33.7	41.3	8.7	7.6	15.3

[1]College of American Pathologists serum Calibrator set

EXAMPLE 2

This example illustrates the separation between cholesterol levels that can be obtained through placement of an additional reagent of the detectable signal reagent system, the horseradish peroxidase which participates in the reaction of the hydrogen peroxide intermediate product with the peroxidase substrate to produce a detectable signal, on the strip in the transfer region or in the region between the sample receiving and measurement regions, as compared to in the transport solution. This example illustrates that the results vary depending on the location of the peroxidase enzyme.

In carrying out the assay for cholesterol, the same device and general protocol was used as in Example 1 and illustrated in Fig. 8. The cholesterol conversion reagents were placed on the strip in the sample receiving region.

Three modifications of the device were tested for separation (in mm) between the tri-level CAP calibrators. The design modifications involved the location of the horseradish peroxidase: (A) transport solution (control), (B) transfer region, and (C) region between the sample receiving and measur ment regions.

With the above modified design, curve separation between the 137 and 333 mg/dl CAP calibrators was 12.0mm when the peroxidase is in the transport solution; 23.3mm when the peroxidase is in the region between the sample receiving and measurement regions. This compared with a 4.4 to 7.5 mm, separation between every 100 mg/dl change using the configuration in which the horseradish peroxidase was placed in the transport solution. Overall, there was observed 95.8% and 41.2% increases in separation, respectively, over the full assay range of about 200 mg/dl with the configuration in which the horseradish peroxidase was placed in the transfer region or in the region between the sample receiving and measurement regions, as compared to the configuration in which the horseradish peroxidase was placed in the transport solution. The following table summarizes the results.

TABLE 2

Location of Conversion Reagents	Cholesterol (mg/dl)[1] 137	234	333	Signal Separation (mm) 137-234	234-333	137-333
A. Transport Solution (Control)	34.5	42.0	46.5	7.5	4.4	12.0
B. Transfer Region	32.7	47.0	56.0	14.3	9.0	23.3
C. Region Between Sample Receiving and Measurement Regions	32.2	40.0	49.0	7.8	7.0	16.8

[1]College of American Pathologists serum Calibrator set

It is evident from the above results that a number of substantial advantages accrue with proper placement of the cholesterol conversion reagent and horseradish peroxidase. By providing for placement of the cholesterol conversion reagent and/or horseradish peroxidase on the strip in the transfer region or in the region between the sample receiving and measurement regions, the distance traversed for an incremental value of cholesterol is increased. Thus, higher sensitivity is achieved, while beneficial performance characteristics are obtained.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

Claims

1. A device for determining the cholesterol level in a sample, said device comprising:
   (a) a first bibulous strip comprising a transfer region for transporting a transport medium from a transport medium source,
   (b) a bibulous member comprising a sample receiving region;
   (c) a second bibulous strip comprising a measurement region;
   wherein said sample receiving region is in fluid communication with said transport and measurement regions, or can be moved into fluid transfer relationship with said transport and measurement regions; with the proviso that when said sample receiving region is moved into fluid transfer relationship with said transport and measurement regions, said device further comprises means for moving said sample receiving region from a first site for receiving said sample to fluid transfer relationship with said transport and measurement regions;
   (d) conversion reagent capable of reacting with cholesterol and cholesterol ester to form an intermediate product present on said strip in the transfer region or a region between the sample receiving and measurement regions such that movement of the transport medium through said strip will bring the conversion reagent and cholesterol together to react to form said intermediate product; and
   (e) reagent non-diffusively bound in the measurement region which reacts in the presence of said intermediate product to produce a detectable border related to the cholesterol level in said sample.

2. The device of Claim 1, wherein said conversion reagent comprises cholesterol esterase and cholesterol oxidase and said intermediate product is hydrogen peroxide.

3. The device of Claim 1, wherein horseradish peroxidase is present on the strip downstream from said measurement region.

4. The device of Claim 1, wherein said conversion reagent is in a region between the sample receiving and measurement regions, when said sample receiving region is in fluid receiving relationship with said transport and measurement regions.

5. The device of Claim 1, wherein said device further comprises a capture region on or upstream from the sample region and the measurement region, which capture region diminishes a predetermined portion of reaction of the bound reagent in the measurement region.

6. The device of Claim 1, wherein said peroxidase substrate is a leuco dye.

7. The device of Claim 1, wherein said device is a single bibulous strip.

8. The device of Claim 1, wherein said device comprises a single bibulous strip comprising the transport region and the measurement region separate from said sample receiving region.

9. A device for determining the cholesterol level in a sample, said device comprising:
   (a) a first bibulous strip comprising a transfer region for transporting a transport medium from a transport medium source,
   (b) a bibulous member comprising a sample receiving region;
   (c) a second bibulous strip comprising a measurement region;
   wherein said sample receiving region is in fluid communication with said transport and measurement regions, or can be moved into fluid transfer relationship with said transport and measurement regions; with the proviso that when said sample receiving region is moved into fluid transfer

relationship with said transport and measurement regions, said device further comprises means for moving said sampl receiving region from a first site for receiving said sample to fluid transfer relationship with said transport and measurement regions;

(d) conversion reagent comprising cholesterol esterase and cholesterol oxidase capable of reacting with cholesterol and cholesterol ester to form an intermediate product present on said strip in a region between the sample receiving and measurement regions such that movement of the transport medium through said strip will bring the conversion reagent and cholesterol together to react to form said intermediate product; and

(e) reagent non-diffusively bound in the measurement region which reacts in the presence of said intermediate product to produce a detectable border related to the cholesterol level in said sample.

10. The device of Claim 9, wherein said device further comprises a capture region on or upstream from the sample region and the measurement region, which capture region diminishes a predetermined portion of reaction of the bound reagent in the measurement region.

11. The device of Claim 10, wherein said capture region is upstream from said conversion region and comprises a reactant capable of reacting with hydrogen peroxide.

12. The device of Claim 9, wherein said sample receiving region is out of fluid transfer relationship with said transport and measurement regions and further comprising means for moving said sample receiving region from a first site for receiving said sample to fluid transfer relationship with said transport and measurement regions.

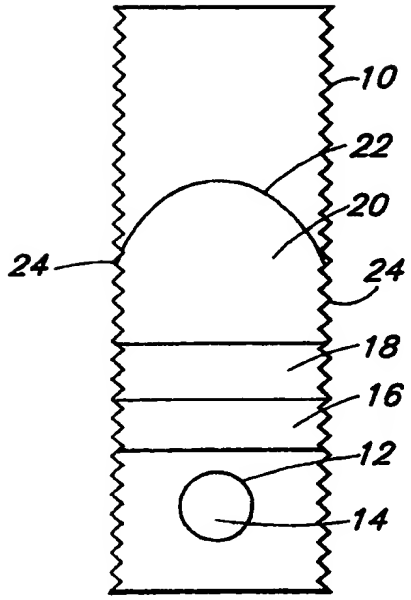

FIG. 1

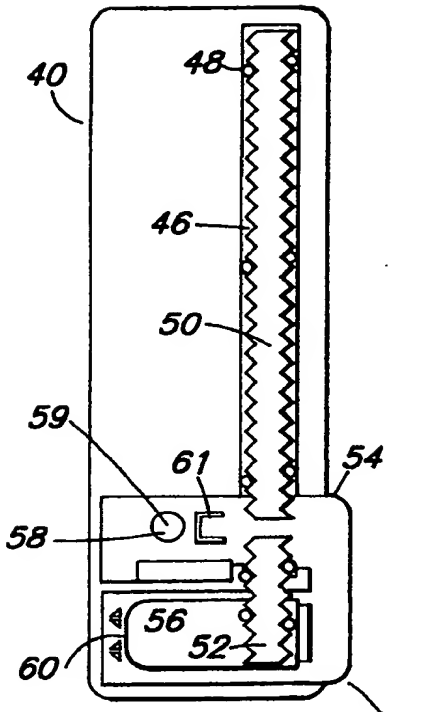

FIG. 2

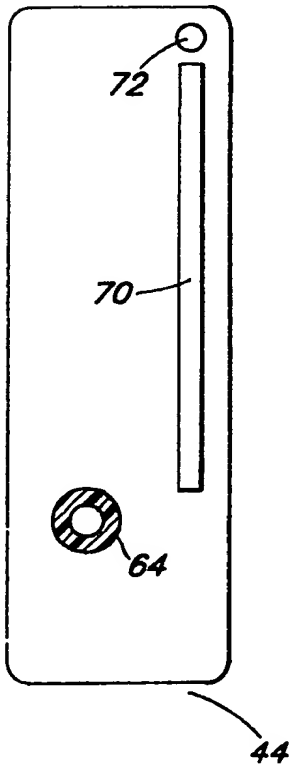

FIG. 3a

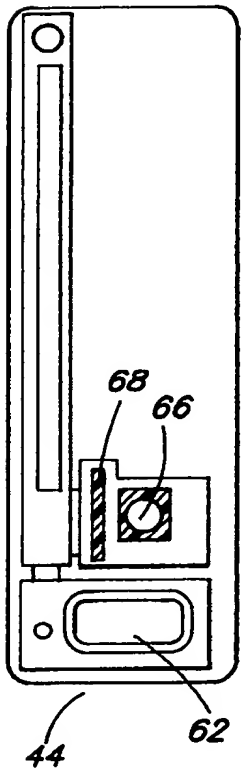

FIG. 3b

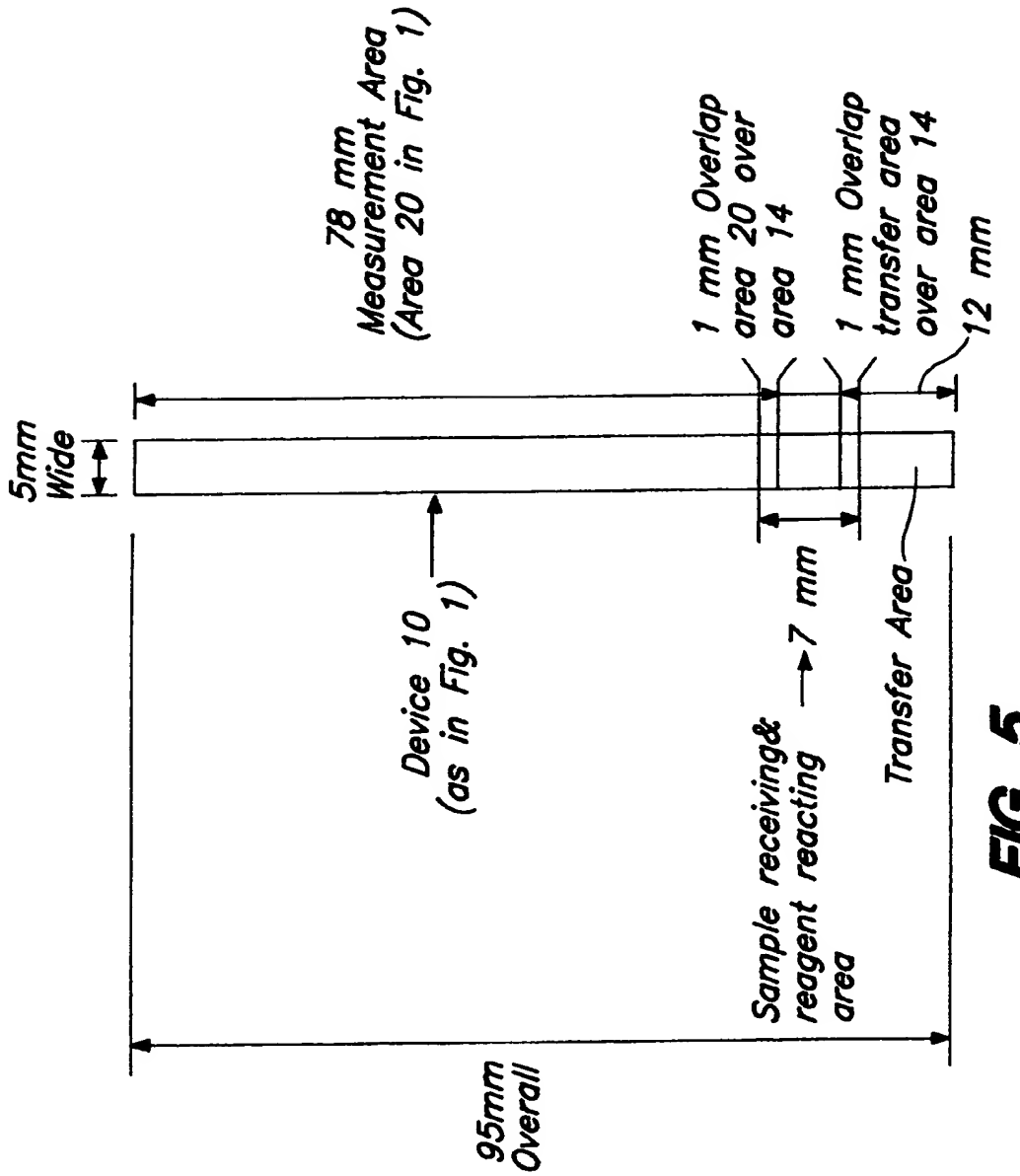
78 mm
Measurement Area
(Area 20 in Fig. 1)
1 mm Overlap
area 20 over
area 14
1 mm Overlap
transfer area
over area 14
12 mm
5mm
Wide
Device 10
(as in Fig. 1)
Sample receiving&
reagent reacting
area
7 mm
Transfer Area
95mm
Overall

FIG. 5

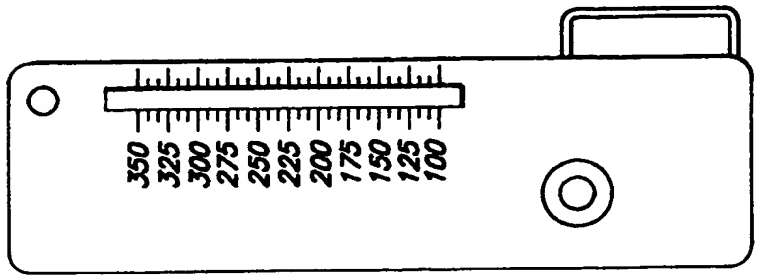
350
325
300
275
250
225
200
175
150
125
100

FIG. 4

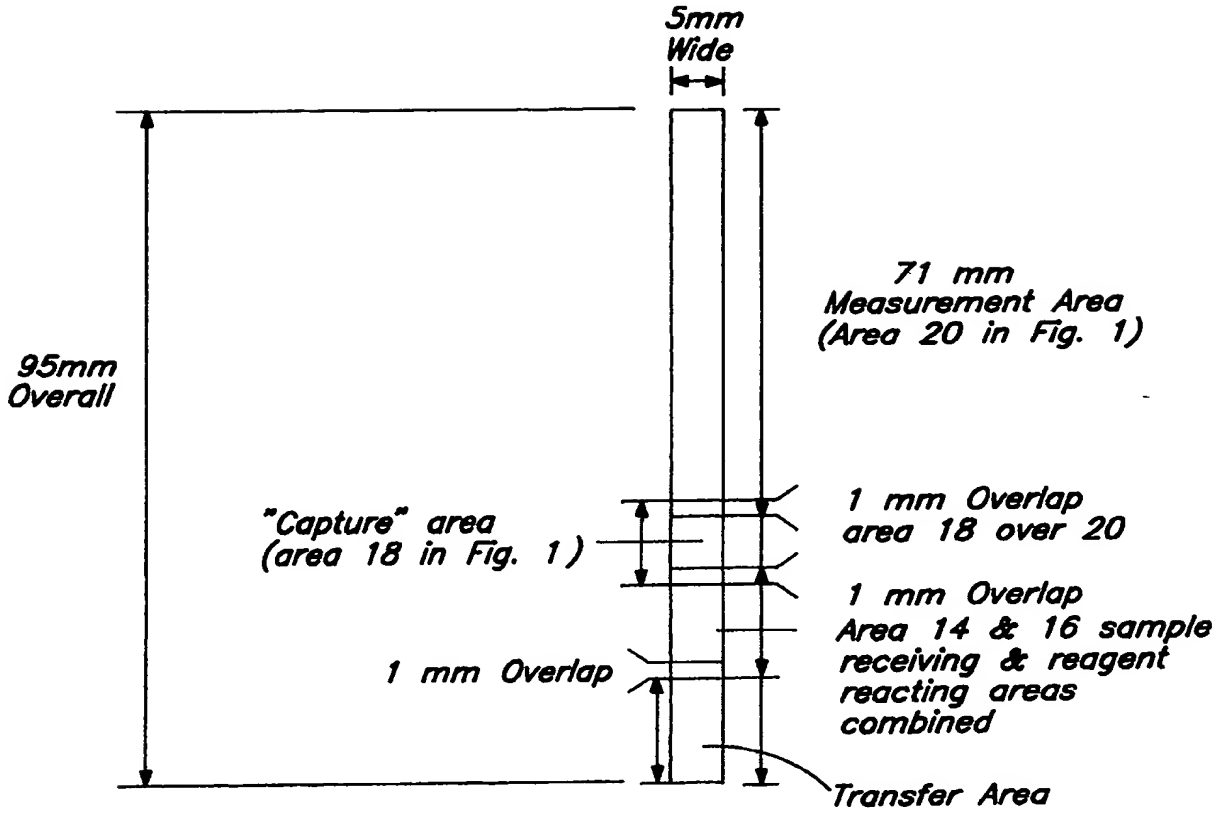
5mm
Wide
71 mm
Measurement Area
(Area 20 in Fig. 1)
95mm
Overall
"Capture" area
(area 18 in Fig. 1)
1 mm Overlap
area 18 over 20
1 mm Overlap
Area 14 & 16 sample
receiving & reagent
reacting areas
combined
1 mm Overlap
Transfer Area

FIG. 6

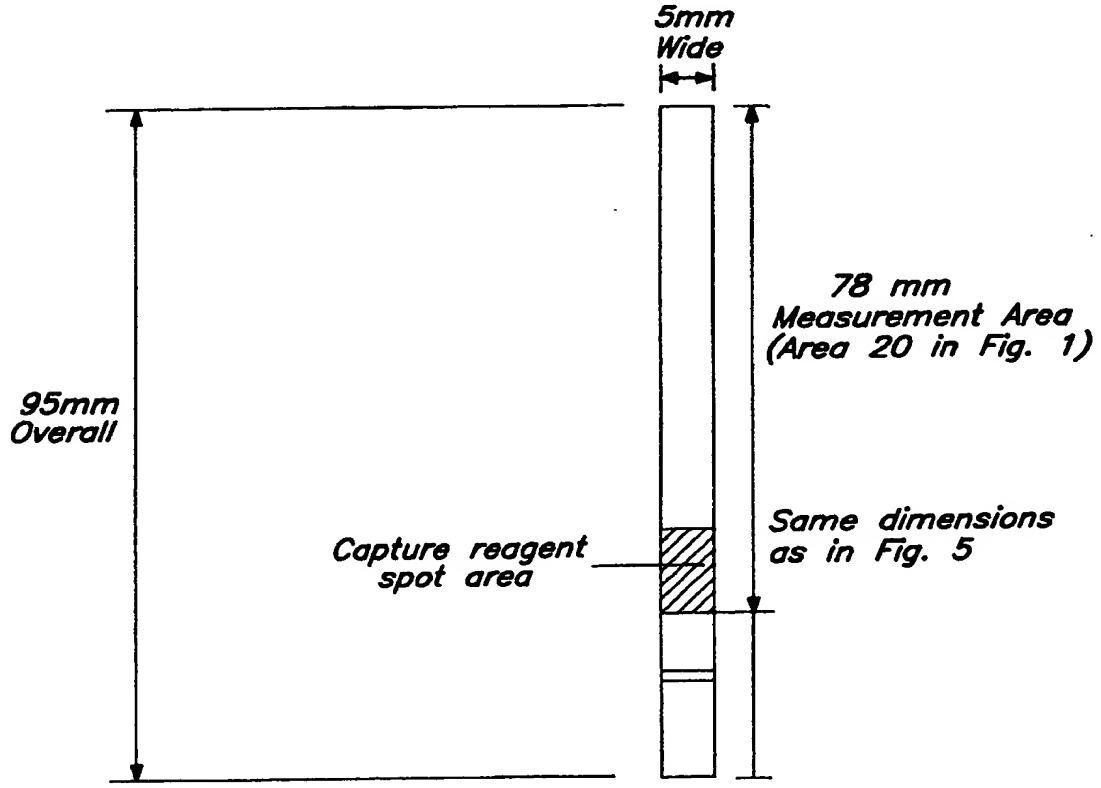
5mm
Wide
78 mm
Measurement Area
(Area 20 in Fig. 1)
95mm
Overall
Same dimensions
as in Fig. 5
Capture reagent
spot area

FIG. 7

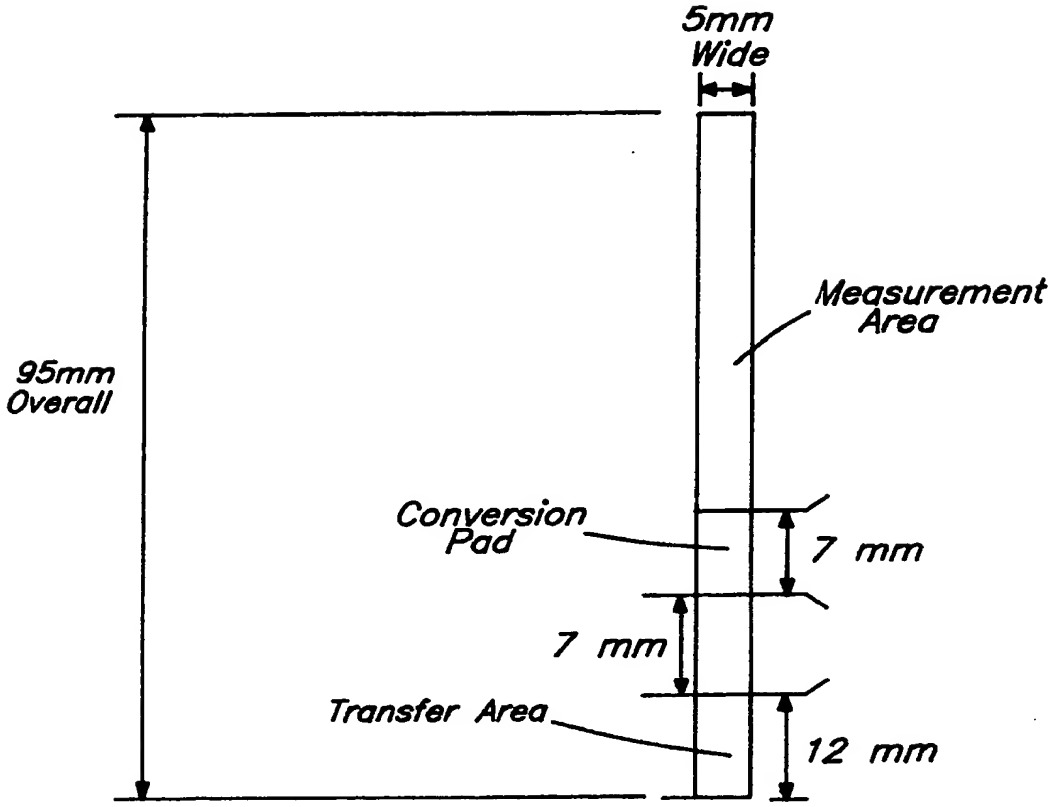
5mm
Wide
Measurement
Area
95mm
Overall
Conversion
Pad
7 mm
7 mm
Transfer Area
12 mm

FIG. 8